Screen design for *Borrelia burgdorferi* lipoprotein secretion pathway components using periplasmic protein capture and suppressor mutations

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Abstract

Borrelia burgdorferi infection, aka Lyme disease, is one of the most prevalent vector borne illnesses in the world. With an estimated 300,000 infections per year in the United States alone, this pathogen places a large burden on the health care system, as well as causing debilitating symptoms in the individuals who are not adequately treated for the disease in a prompt manner. While the normal life cycle is based upon transmission by ticks from small mammals to large mammals, mainly deer, human infection results in a dead end for *Borrelia*. The maintenance of this life cycle is a key feature of *Borrelia*, and understanding its ability to live in two contrasting environments gives researchers a framework for understanding the disease.

Genes for bacterial lipoproteins (lipid modified proteins) make up a large portion of *Borrelia* species' genomes and play important role in the infection/transmission cycle. While lipoprotein modification and localization is understood in several bacterial species, such as *Escherichia coli* and *Bacillus subtilis*, a few key components of the lipoprotein localization pathway are missing in *Borrelia*. Furthermore, the signals involved in proper lipoprotein sorting are less clear than in other species. Based on earlier studies in the laboratory, there are two proposed pathway components that are missing from a minimally functioning lipoprotein sorting machinery in *Borrelia*: a lipoprotein chaperone, which stabilizes the newly translocated lipoprotein in the outer membrane, and a lipoprotein "flippase", which allows lipoproteins to pass through the outer membrane to the surface. To facilitate identification of these two hypothetical pathway components in functional screens, we embarked on two approaches. First, a mutational analysis of the complement binding protein CspA was performed with the aim to generate a subsurface mutant of CspA. CspA is a

complement factor H binding protein that renders the bacteria serum-resistant. The resulting subsurface mutant would provide a basis for a functional suppressor screen, selecting for pathway mutants that are capable of transporting the mutant CspA to the surface. Additionally, the analysis would reveal the amino acids involved in localization of this lipoprotein, adding to the existing dataset with other *Borrelia* lipoproteins. In a parallel secondary approach, two separate binding proteins (Calmodulin and LysM) were setup to bind their respective targets (Calmodulin Binding Peptide and peptidoglycan) within the periplasm of *B. burgdorferi*, in an effort to stall the localization process.

Based on our earlier work with *Borrelia* surface lipoproteins, a series of deletions within the intrinsically disordered N-terminal CspA tether were generated. Deletions of both the first and second halves of the tether peptide in CspA failed to yield a subsurface phenotype. Starting with a second-half tether deletion, escalating stepwise 4-amino acid deletions in an N-terminal direction were made. Still, no subsurface CspA mutant was recovered, with the first 4 CspA tether amino acids still supporting the lipoprotein's surface exposure. Additionally tether swapping with a subsurface localizing lipoprotein tether yielded no subsurface CspA. Second, two protein trapping methods were attempted. These were designed to stall the interaction between pathway components and lipoproteins inside of the periplasm. The introduction into the bacterial periplasm of the mammalian binding protein Calmodulin and surface sorting lipoprotein fused to a tag derived from a Calmodulin Binding Peptide yielded no transformants in single or multiple plasmid systems. Plasmids encoding for each of the individual components could be readily transformed separately. Alternatively, introduction of a fusion protein that links a surface red fluorescent

protein reporter lipoprotein to a native peptidoglycan binding peptide tag yielded significant filamentous growth as well as overall growth defects in *E. coli*, and no *B. burgdorferi* transformants were recovered.

Acknowledgements

I would like to thank the committee for being flexible in defending this thesis on such short notice, the members of the Zückert lab for assistance and guidance in performing experiments, and Dr. Wolfram Zückert for his patience awaiting my completion of this thesis.

Lastly, I would like to thank my wife Ashley Eusey for her support, patience, and artistic ability, without which I likely would not have completed this work.

Table of Contents

1. Lyme Disease

Since their discovery in 1905, spirochetes have been prominent pathogens in the field of human health (Tampa et al, 2014). The venereal disease syphilis, caused by *Treponema pallidum*, at the time changed marriage laws by requiring a blood test prior to applying for a marriage license (Quetal, 1990). Another spirochetal genus, *Leptospira*, causes leptospirosis (i.e. Weil's disease, black jaundice) and is one of the most prominent infections across the globe, with an estimated 14 cases per 100,000 people infected per year (Haake and Levett, 2015). Due to advances in medicine, *Treponema pallidum* is easily treatable with antibiotics and easily preventable using safe sex practices. Although quite deadly if not diagnosed soon enough, antibiotics and sanitary living conditions can prevent diseases caused by *Leptospira*.

While syphilis and leptospirosis have been known for more than a century, another spirochetal disease discovered in Lyme, Connecticut has been emerging as a widespread human health concern. Commonly referred to as Lyme disease, the disease is caused by infection with *Borrelia burgdorferi* (Burgdorfer, Barbour, et al, 1982). Since being discovered in 1976 due to an outbreak of misdiagnosed juvenile arthritis, the disease has grown from relative obscurity to being the most common vector borne illness in Europe and North America (Mead, 2015) with an estimated 300,000 cases per year in the United States alone (CDC, 2014). The clinical manifestations of Lyme borreliosis have been documented well before the link to the spirochete was discovered: Afzelius describing erythema migrans, a growing target shaped rash, (Afzelius, 1921) and Garin and Bujadoux describing neurological symptoms after a tick bite (Garin and Bujadoux, 1922). However, the link between the neurological and accompanying symptoms associated with the cases described by Garin and Bujadoux and *Borrelia* have been recently called into question (Wormser and Wormser, 2016).

Due of the meteoric rise of Lyme disease cases in only 40 years, and the difficulty in correctly diagnosing the disease, there are a number of misconceptions about Lyme disease in the public. Many of these misconceptions are based on how *B. burgdorferi* is able to cause a persistent infection. This misconception is birthed from the symptoms of Lyme disease potentially lasting years after elimination of the pathogen. Whether or not the continuing symptoms are caused by a continuing infection or damage caused by the prior infection has been hotly debated amongst medical professionals (Halperin, 2015). When diagnosed early, a course of antibiotics can completely cure the bacterial infection with no sign of continuing symptoms (Steere et al, 1994). However, if misdiagnosed or undetected, the disease can successfully evade the immune system using a series of genetic programs that allow for antigenic variation of a lipoprotein (a type of post-translational lipid modification) (Zhang et al, 1997) (Norris, 2014) as well as complement evading proteins known as CRASPs (Kraiczy et al, 2001).Without appropriate treatment *Borrelia burgdorferi* will continue causing Lyme disease and set up a persistent infection. Continuing infection will include symptoms of arthritis, malaise, fatigue, neurologic impairments, and in severe cases endocarditis (Steere et al, 1977). Upon diagnosis of Lyme borreliosis, treatment of the patient with penicillin or doxycycline will ensure the bacteria are eliminated from the body (Nocton et al, 1994). Antibiotic resistant Lyme disease is rare (Steere et al, 2004). A recent study confirming the existence of drug-tolerant *Borrelia* in humans found that these "persister" cells were not antibiotic resistant and had not acquired heritable antibiotic resistance traits. These drug-tolerant *Borrelia* are not replicating or metabolically active and are found to be morphologically distinct from log phase *Borrelia* (Sharma et al, 2015). This study establishes that *B. burgdorferi* can form a drug persister state in response to many different antibiotics *in vitro*, however the

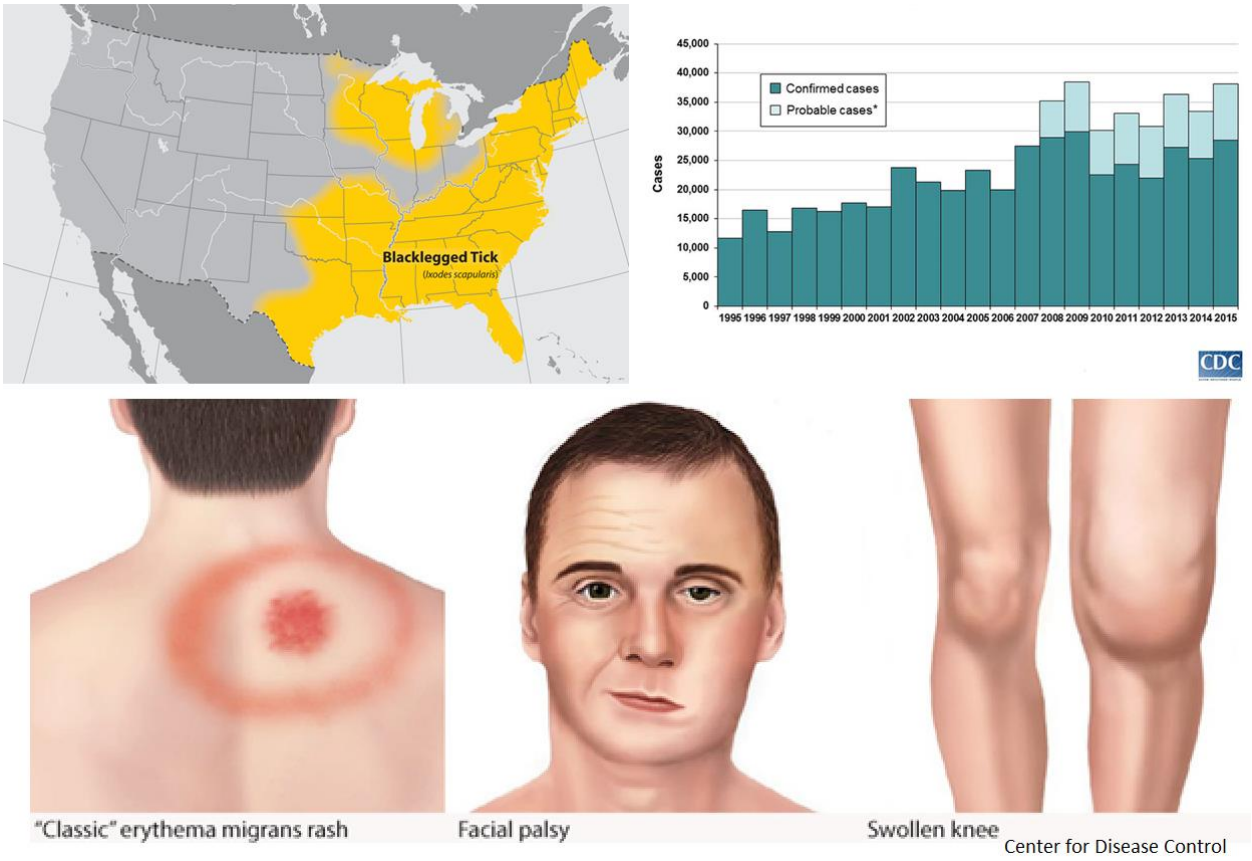

Figure 1. Lyme Disease. *Borrelia burgdoferi* is the causative agent of the tick transmitted illness known as Lyme disease, or Lyme borreliosis. The major carrier of Borrelia Burgdorferi is Ixodes Scapularis which has a natural range that covers the majority of the eastern United States. Since its description in 1982 the number of reported Lyme disease cases has grown to over 30,000 cases per year with estimated cases rising to over 300,000 cases per year according to CDC reports. While not all symptoms are present in every case of Lyme disease, common symptoms include a growing target shaped rash called erythema migrans, facial (Bell's) palsy, and arthritis in major joints.

formation of persister cells does not lead to chronic illness in healthy patients due to immune clearance, but immunocompromised individuals are at risk for chronic *Borrelia burgdorferi* infection. Approximately 10% of US cases continue to suffer from arthritis after resolution of the infection due to damage already done during the infection (Steere, 1987). Additionally other persistent, co-infective, tick-borne pathogens as well as the detrimental effects of a course of broad spectrum antibiotics can continue past clearance of the bacteria (Ettestad, 1994). There are currently no vaccines available to humans for Lyme disease although there is at least one in the later stages of development (Comstedt, 2014).

2. Life Cycle

Although most notable for causing human Lyme borreliosis, *Borrelia burgdorferi* evolved separate from human interaction and has become completely dependent on the life cycle of ticks in the *Ixodes* genus (Kurtenbach, 2002). *Borrelia* starts the cycle in a reservoir species. Reservoir species are organisms which can carry a pathogen with little to no detriment to themselves or their reproduction, and can also be a site for multiple vector transmissions without detrimental immune surveillance (Donahue, 1987). The reservoir species for *B. burgdorferi* are typically smaller animals including rodents, lizards, and birds that are available for the larval form of *Ixodes* ticks to gain access to a blood meal (Steere et al, 2004). It has been proposed that birds are responsible for the quick dissemination of the disease over the past 40 years from strictly in New England to being found in half of the United States, although the reduction of farmland and increase in predator-less habitat in deer (Steere, 1994) is also hypothesized play a role in the increased geographic scope of the disease. Since *Ixodes spp.* are not specific in their host range, there are a large number of dead end hosts for *B. burgdorferi* (Margos, 2011). For *B. burgdorferi*, dead end hosts are either large mammals that can be infected, but are not capable of transmitting the

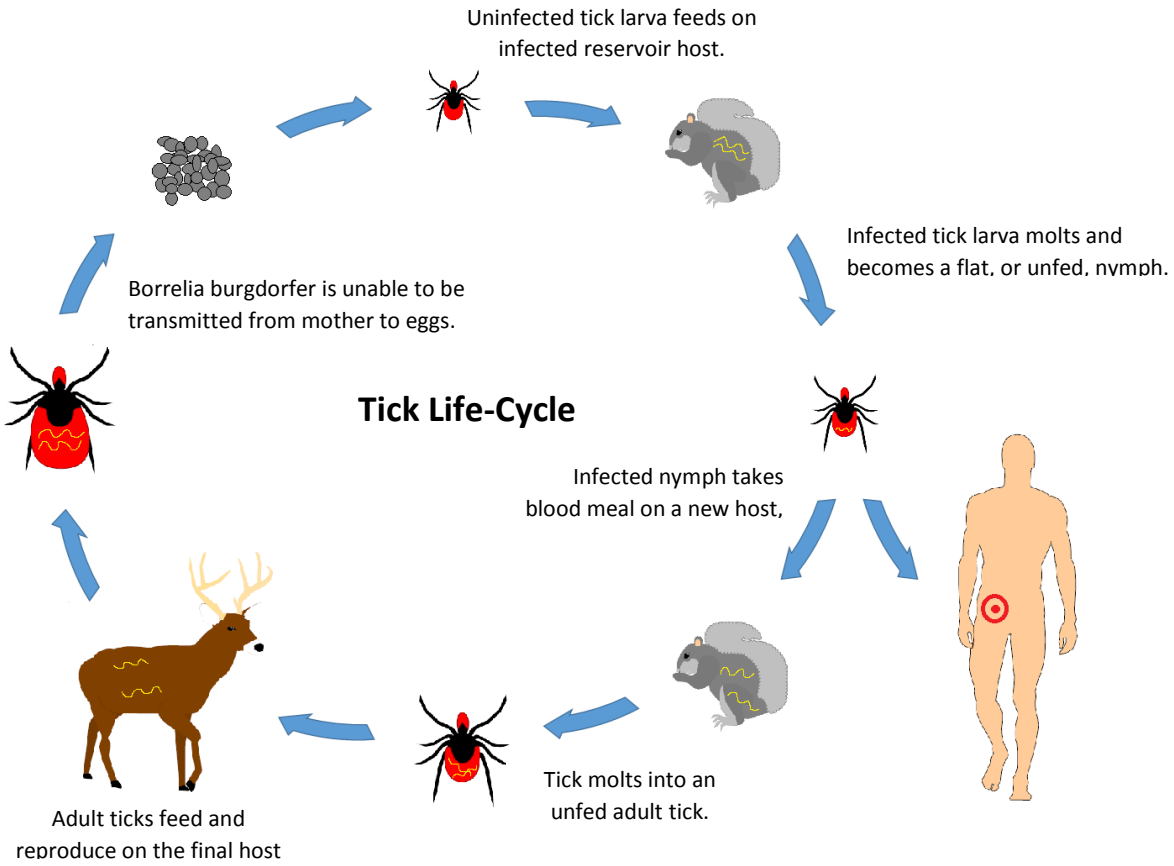

Figure 2. Tick Enzoonotic Cycle. The Ixodes tick will undergo three blood meals in its lifecycle. Each blood meal will support either a transformation from larvae to nymphs to adults, or begin the reproductive cycle. Unlike relapsing fever and other tick-borne diseases, *Borrelia burgdorferi* is not transmitted transovarially (Rollend et al, 2013) and the larvae are born uninfected. *Borrelia* is acquired from an infected reservoir host during larval feeding. In the early summer larva is hatched and attempts to take its first blood meal in the late summer. Upon feeding the tick will drop to the ground and overwinter until May. As the larva is molting, the spirochete waits the long months until the nymph can undergo feeding. Once molted, the nymph has a much larger feeding range that still includes *Borrelia*'s reservoir species but also includes the dead end hosts including human. The nymph tick will attempt to take a blood meal in the late spring/early summer. If successful fed nymph will then grow into an adult over the next winter and reproduction will occur on a deer the following spring (Anderson and Magnarelli, 1980).

pathogen back to the tick (humans fall in this category), or are animals which have a bolstered immune defense to the pathogen (Radolf et al, 2012). Given that *Ixodes* has such a large host range and are able to host a large range of pathogens from viruses to fungi to bacteria to protozoa, the tick-*Borrelia* interaction plays a large role in the progress of the disease (Jongegan and Uilenberg, 2004).

2.1. Tick Uptake, Survival and Transmission

Then a pathogen enters the tick through the mouth during a blood meal, the goal of the pathogen is to survive in the tick environment until retransmission. While in the midgut, ovaries, hemocoel, salivary glands or other immune deficient locations, the pathogen awaits until another blood meal can be taken and can be expelled through the salivary glands or until reproduction occurs and the pathogen is transmitted transovarially (Hajdusek et al, 2013). While theoretically the pathogen could move to any and all of these locations, as determined by their migration abilities and ability to deal with the tick immune system, the most likely place for a pathogen to reside is in the midgut of the tick. The midgut has limited digestive capabilities, an abundance of nutrients, and redox balancing agents that not only allows for commensal bacteria to live, but also for pathogens such as *B. burgdorferi* to survive the months between feedings (Hajdusek et al, 2013). While the midgut is a favorable environment for organisms in which to dwell, there are still challenges to overcome. Ticks do have antimicrobial peptides, hemocidins, and defensins to keep microbes from overrunning the midgut (Kopacek et al, 2010). However, these compounds are not species specific attacks, but generalized against Gram (+) and Gram (-) bacteria to which the tick-borne pathogens and commensal organisms have found defenses or avoidance measures. *Borrelia* are neither classified as Gram (+) or Gram (-), but are classified as diderms (Aberer and Durray, 1991). During the meal in which *Borrelia* is transferred from reservoir to the tick, *Borrelia* will flip a number of

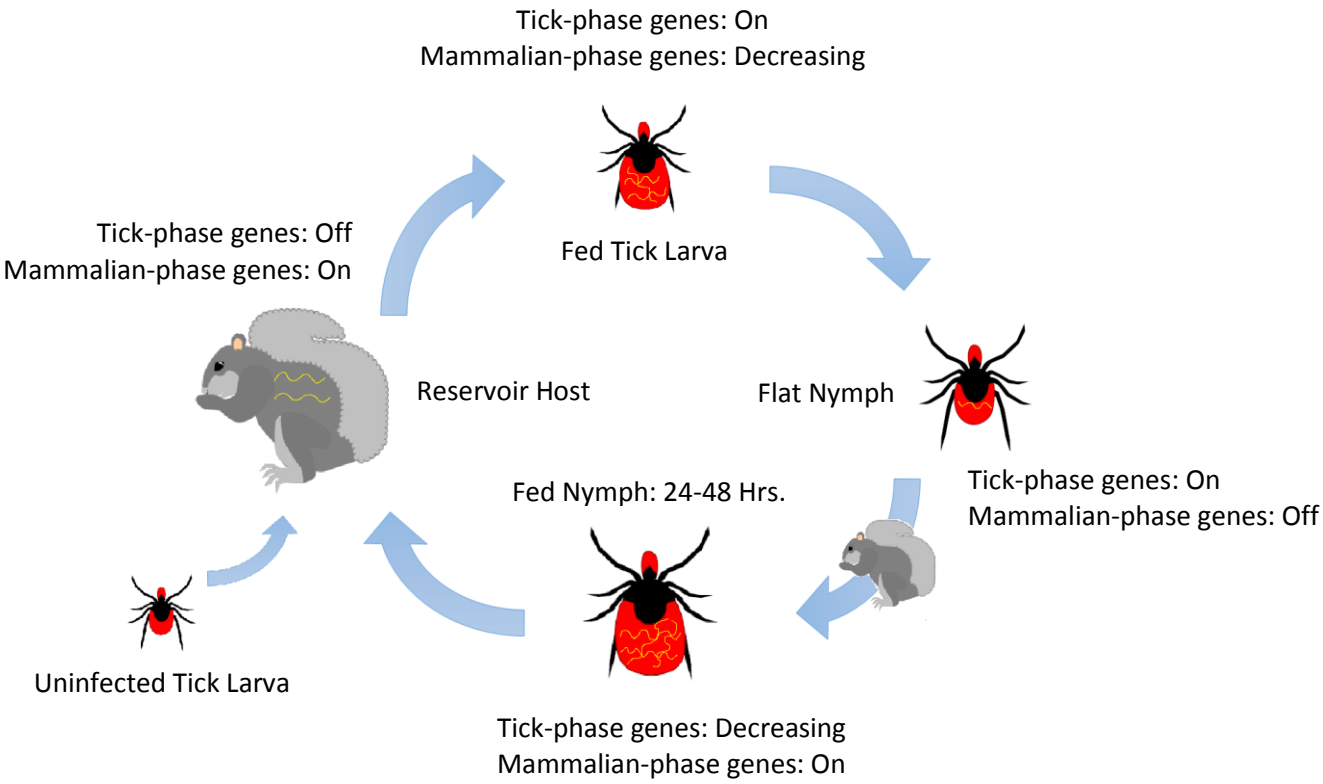

Figure 3. *Borrelia* Genetic Control. *Borrelia burgdorferi* must survive in two very different environments and the transitions between them. To start *Borrelia* must protect itself from the threats of the reservoir species, typically a rodent, but it can also be found in birds and lizards. When the unifected tick takes a blood meal *Borrelia* will activate its tick phase genes in response to the saliva secreted by the tick. As *Borrelia* makes its way into the tick midgut, the tick environment will trigger the necessary genes for *Borrelia* to survive the coming months as the nutrients from the blood are depleted and mammalian phase genes are slowly turned off. After the tick molts into the nymph another blood meal will be taken. *Borrelia* will activate its mammalian-phase genes in response to heat and blood components/nutrients. This will cause and increase in proliferation and cause the migration of *Borrelia* to the new host.

genetic switches to survive within the midgut of the tick. These changes in gene expression allow the bacteria to survive in three different environments within the tick (fed, fasted, and transmitting) as well as the mammalian host environment (Tsao, 2009).

2.1.1. Acquisition and Replication

When the tick bites the reservoir host, the tick's salivary glands will release a number of pro-inflammatory factors, anticoagulants, and antimicrobial peptides into the host (Hajdusek et al, 2013). This allows blood to flow freely from host to tick. It is not completely known which factors play a role in *Borrelial* migration to the tick bite, but there is evidence that the uptake of *Borrelia* is not completely random (Moriarty et al, 2008). *Borrelia* can be detected in the tick after 24 hours before significant amounts of blood have been imbibed (Radolf et al, 2012). As the blood meal is being taken, the *B. burgdorfe*ri lipoprotein Bbe31 interacts with the tick protein Tre31 to disseminate from the mouth of the tick throughout the intestinal tract (Zhang et al, 2011). Entering the digestive tract, *Borrelia* must face the challenge of adhering to the midgut and preventing movement to the harsh environment of the hemocoel (Hajdusek et al, 2013). This is solved by the lipoprotein OspA binding to the midgut protein TROSPA (Target Receptor of OspA), allowing *Borrelia* to stay and compete for nutrients with the host (De Silva et al, 1996) (Pal et al, 2000). OspA, while highly immunogenic, also serves as a protective agent against antibodies. OspA is primarily expressed inside the tick vector, suggesting that the protection offered by OspA is only useful in the absence immune surveillance, although OspA may have a mammalian function as it is expressed in the late mammalian phase (Tilly et al, 2016). Once established in the tick, *Borrelia* uses the available resources from the blood to replicate (Li et al, 2007) while the changed environment triggers the activation of tick phase genes (Caimano et al, 2011). To deal with the changing nutritional and metabolic needs of the tick vector, *B. burgdorferi* must change the genes

transcribed and transcribed to better fit their metabolic needs and adapt to their environment. These genes are controlled in response to lowered nutrient levels by metabolic regulatory cascades. The histidine kinase cascade Hk1-Rrp1 responds to lowered nutrient levels and makes c-di-GMP (Kostick et al, 2011). In addition, the SpoT/RelA homologue Rel_{bbu} regulates (p)ppGpp levels in response to lowered nutrients (Drecktrah et al, 2015). The buildup of c-di-GMP as well as (p)ppGpp levels leads to the activation and transcription of tick phase promoters and sigma factors. The transcription and translation of tick phase genes allow the bacteria to survive for an extended period of time with limited nutrients as the tick proceeds through its life cycle to become a flat nymph.

2.1.2 Survival

As the *Ixodes* larva finishes its blood meal, it will drop from the primary host to the ground and molt into a nymph over the course of a few months. After only a few weeks the nutrients in the tick are depleted and *Borrelia* must wait out the winter in the low nutrient environment of the unfed nymph. *Borrelia burgdoferi* must lower its nutritional requirements as well as scavenge any nutrients that are available in order to survive. In this environment *B. burgdorferi* will stop replication and become extremely sluggish in its movement (Pal, 2010), but it does not become dormant and stop all transcriptional and translational activities (Dunham-Ems et al, 2009). Certain tick phase genes are still expressed in relatively high numbers. During starvation, the genes associated with glycerol metabolism are up regulated to provide a carbon and energy source for *Borrelia* (Pappas et al, 2011). The oligopeptide transporter *oppA* is also up-regulated, under the control of $Rel_{bbu,}$ to provide amino acids because *Borrelia* is incapable of synthesizing them (Drecktrah et al, 2015) (Gherardini, 2010). In addition to metabolic changes, the cell surface of

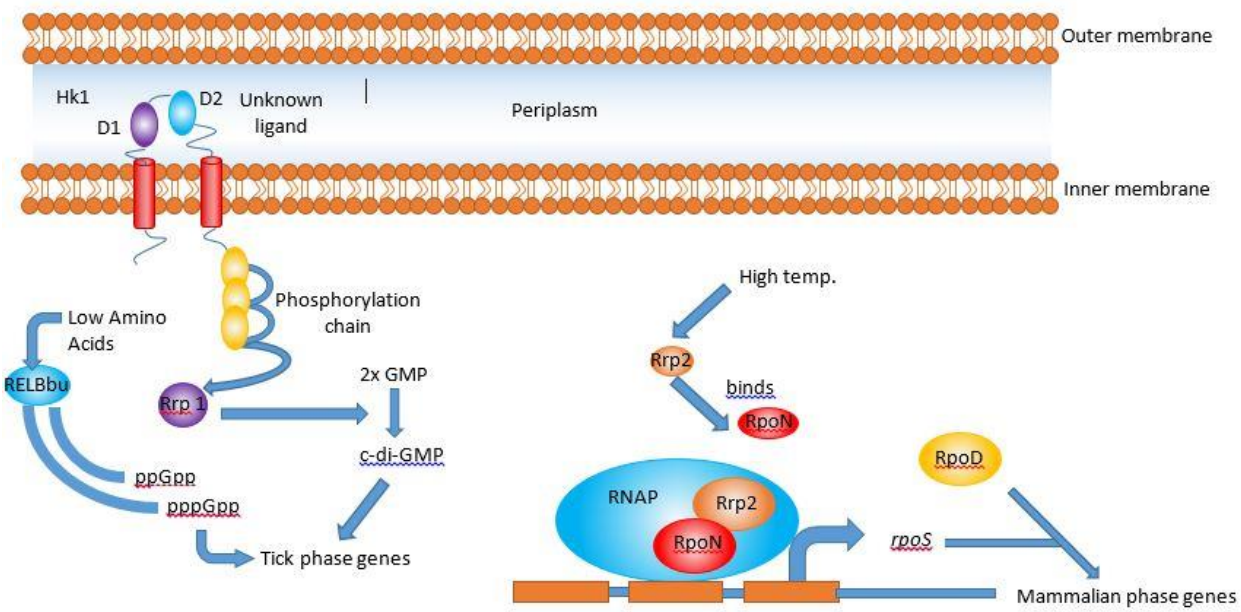

Figure 4. **Signaling Pathways.** Genetic control of *Borrelia burgdorferi* is dependent on the use of master regulator sigma factors that are regulated by the environment they are currently living in. Low amino acids induce tick phase genes through the metabolic enzyme RELBbu which makes (p)ppGpp. A yet undiscovered stimulus is responsible for activating tick phase genes through the creation of cyclic di-GMP by phosphorylated Rrp1. High temperature, presence of blood, and nutrients lead to the up regulation of sigma factors RpoS and RpoD through the Rrp2/RpoN pathway, which control the activation of mammalian phase genes (modified from Radolf et al, 2012)

Borrelia changes to increase interaction with the extracellular matrix (Drektrah et al, 2015). While there have been micro arrays performed in similar conditions to the unfed tick, many of the genes that are affected by starvation have an unknown function. This makes understanding *Borrelia's* ability to survive in such conditions difficult (Pal, 2010).

2.1.3. Transmission to Host

Once the infected tick has molted and matured to the nymph from, it seeks another blood meal. The tick now has a larger host range due to increased size and can deliver *Borrelia* to a wider range of species. This explains why nymph ticks transmit the majority of the disease involved in human cases (Tilly et al, 2008). As the tick begins to engorge itself on the new host's blood, *Borrelia* must now defend itself from a number of threats inside the blood, scavenge as many nutrients as it can, and make its way from the midgut to the salivary glands to be excreted into the new host. The change in temperature from being on a warm-blooded host activates the Rrp2–RpoN–RpoS pathway as well as the sigma factor RpoD to prepare *Borrelia* for the mammalian phase of infection as well as repress tick phase genes (Ojaimi et al, 2003, Radolf et al, 2012). Rrp2, which is activated by autophosphorylation in the presence of acetyl phosphate, mediates the opening of the *rpoS* promoter and activates the sigma factor RpoN. This Rrp2-RpoN complex then activates the transcription of *rpoS*, which, when translated, acts as an alternative sigma factor during the mammalian host phase (Hu et al, 2010). Temperature, the presence of blood nutrients, and blood immune factors seem to be required for the complete transcriptional switch from tick phase genes to mammalian phase genes (Tokarz et al, 2004). While it is known that *Borrelia* must move from the midgut to the salivary glands (Schwan et al, 1995) for eventual exit from the salivary gland into the mammalian host (De Silva and Fikrig, 1995), the mechanism of travel from the midgut to the salivary gland has yet to be determined. Once in the host, the bacteria are in a nutrient rich, yet

hostile, environment. Here they must successfully evade the immune system whilst awaiting another tick bite to propagate the species. Although adult ticks can be infected by and transmit *Borrelia*, the processes by which *B. burgdorferi* survives, replicates and is transmitted to a new host is very similar to the nymphal form.

Among the proteins that are upregulated in response to blood are a family of proteins called Complement Regulator-Acquiring Surfacing Proteins (CRASPs). CRASPs are an important part of immune evasion and immune defense that *Borrelia* has evolved in order to persist within the fed tick and the mammalian hosts (Kraiczy et al, 2001). The surface lipoprotein CspA (CRASP-1) is highly expressed in the anticipation of this blood meal to defend itself from mammalian complement (Hammerschmidt et al, 2014). As the blood meal arrives, CspA dimers and the lipoprotein OspE independently bind to Factor H. The role of Factor H in mammals is to bind to normal "self" cells within the host to prevent autolysis using the Alternative Complement Pathway (Kraiczy, 2004) (Hellwage, 2001). When CspA binds to Factor H, the complement system will overlook *Borrelia* and prevent complement lysis. CspA also binds to complement components C7-C9 making it difficult for the Membrane Attack Complex to form on the surface (Hammerschmidt et al, 2014). Recently, the lipoprotein BBK32 has been shown to bind the complement factor C1. Complement factor C1 is a part of the Classical Complement Pathway and is recruited by IgG and IgM antibodies to the binding site. BBK32 binds C1 and the C1r subunit to prevent antibody mediated lysis (Garcia et al, 2016). The ability of BBK32 to bind complement also demonstrates the ability for proteins to have multiple roles, as BBK32 was first identified as a fibronectin binding protein (Li et al, 2006).

Lipoproteins such as OspC, CspA, OspE, and BBK32 are important in overcoming the innate immune system. OspC has been determined to prevent phagocytosis by macrophages, a crucial

step in passing through the first barrier of the skin (Carrasco, 2015). The lipoprotein VlsE is a part of the antigenic variation system that allows *Borrelia* to avoid the adaptive immune response using random recombination events with silent gene cassettes. While each one of these variable surface proteins may or may not be antigenic, the randomization of the antigen throughout the mammalian life cycle gives the *Borrelia* infection as a group an advantage over the antibody response. It also continuously delays a detrimental immune response as the expressed protein is varied (Norris, 2014). Along the same lines, *Borrelia* will vary the expression of the surface proteins VlsE, OspA and OspC in response to the host's immune surveillance. Indeed, VlsE is expressed as OspC is downregulated, and OspC is produced only during early mammalian infection when OspA is downregulated (Ohnishi et al, 2001). Not only can *Borrelia* vary expression of proteins to avoid immune surveillance, but it can also modify the proteins themselves to achieve a greater level inconspicuousness (Tilly, 2016). Overall, these examples show that surface lipoproteins are crucial to the life cycle of *Borrelia* as it moves from host to tick and eventual return to mammalian host.

3. *Borrelia burgdorferi*

3.1. Genome

Borrelia burgdorferi has a uniquely structured, fragmented, and complex genome, especially considering its relatively small genome size. The main chromosome is linear, with covalently closed ends (telomeres), and approximately .91 megabases in length (Fraser et al, 1997). This linear main chromosome encodes for 846 predicted intact proteins, including the majority of metabolic proteins and all but one protein (encoded by the circular plasmid or "minichromosome" cp26) that is required for growth in culture. Close to 70% of the proteins on the main chromosome have homologues with either known or unknown bacterial proteins, leaving approximately 250 coding sequences that appear unique to *Borrelia*/*Borrelia burgdorferi* (Fraser et al, 1997). Like

the various other species that make up the *Borrelia* genus, *B. burgdorferi* carries a plethora of circular and linear plasmids (Barbour, 1988). As exemplified by the lipoproteins discussed above, these plasmids carry a variety of different genes required for various facets of the *Borrelia* life cycle. While each *Borrelia* species contains a highly similar chromosome, up to 93% nucleotide identity excluding the telomeric regions (Glockner, 2006), the plasmid content of these *Borrelia* species are highly variable in total number of plasmids and in the ratio of linear plasmids to circular plasmid. For the *Borrelia burgdorferi* sensu stricto laboratory strain B31, there are 12 linear plasmids and nine circular plasmids with a size range of 9 to 62 kilobases (Fraser et al, 1997). While most species of bacteria contain circular genomes and circular plasmids, *Borrelia* species' genomes require telomeric ends to their linear DNA segments and behave more similarly to eukaryotic genomes in terms of replication and homeostasis.

3.2. Metabolism

The metabolism for *Borrelia* is very simple, which is expected considering the size of the genome. There are no genes for the TCA (Krebs) cycle, respiratory (electron transport) chain, or for nucleic and amino acid synthesis (Fraser et al, 1997). This makes the bacteria highly reliant on obtaining the basic requirements of life from the surrounding environment. For most bacteria the inability to synthesize some of the basic building blocks of life would be detrimental. However, because *Borrelia*'s life cycle has been adapted to the high nutrient environments it lives in, any ability to synthesize nutrients in such an environment would have been selected out in favor of increased scavenging abilities. While *B. burgdorferi* is an efficient scavenger of nutrients, a unique trait of *Borrelia burgdorferi* is the complete lack of the use of iron as a cofactor. While many bacteria engage in an iron scavenging war with their hosts using bactoferrins, *Borrelia* avoids this process

altogether (Posey and Gherardini, 2000). Instead it uses other divalent ions such as manganese (Troxell et al, 2012) and zinc (Nguyen et al, 2013).

Another aspect of metabolism is an organism's ability to break down the chemicals that other organisms employ as a defense mechanism. Many organisms employ a chemical defense that contains reactive oxygen species. Without catalase, *Borrelia* is susceptible to oxidative stress, and is restricted to low oxygen concentrations as to avoid oxidative damage and excess free radicals (Fraser et al, 1997) although *Borrelia* does have a manganese based superoxide dismutase (Troxell et al, 2012).

3.3. Cell Structure

B. burgdorferi is classified as a spirochete, but the bacterium is not actually spiraled in shape. While neither truly Gram positive or negative, the diderm rod that makes up *Borrelia* is a flat wave that gives the illusion of a spiraled corkscrew when in motion (Goldstein et al, 1994)(Gupta, 1998). *Borrelia* spp. stain weakly Gram-negative, but their lack of lipopolysaccharide (LPS) on the surface eliminates one of, if not the main marker for immune systems to recognize Gram (-) bacteria. Between the two lipid bilayers, a thin layer of peptidoglycan gives support to the cell, and bundles of flagellin give the cell its shape and ability to move (Charon et al, 2009) (Dombrowski et al, 2009).

4. Bacterial Lipoproteins

Lipoproteins are proteins that have been covalently modified post-translationally with a fatty acid moiety. Bacterial lipoproteins are found outside of the cytoplasm and can be located on either the periplasmic surface of the inner membrane, or on either side of the outer membrane of most bacteria (Wilson and Bernstein, 2015). Lipoproteins perform a wide variety of tasks from

providing envelope structure and stability, and aiding in cellular division, ATP transport, pheromone signaling, virulence, and immune evasion (Kovacs-Simon, 2011).

4.1 Lipoprotein Usage

As membrane-associated proteins, lipoproteins have some key advantages and disadvantages compared to their unlipidated integral membrane protein counterparts, which contain domains that span the entirety of the phospholipid bi-layer. Characteristically, the lipid bilayer moves very quickly in relation to the proteins that are associated with it. By peripherally anchoring the protein via a lipid moiety, the membrane remains more fluid, as compared to anchoring with integral membrane proteins. The presence of lipid rafts (slow moving protein and cholesterol dense regions of the lipid bilayer) is unique to *Borrelia* amongst bacterial species, and the use of cholesterol in their membranes is rare across the bacteria kingdom. The use of lipoproteins in lipid rafts and the preferential localization of lipoproteins to lipid rafts gives an order and structure to the outer membrane (Toledo et al, 2014). With over 120 lipoproteins, *Borrelia* membranes can remain flexible and can withstand the force of the flagellar machinery located in the periplasmic space (Dombrowski et al, 2009). Conversely, integral membrane can act as anchors that give structure and order to the membrane and bacteria as a whole (Kenedy et al, 2012). Lipoproteins bridge the gap between integral membrane proteins and amphipathic protein domains, which are very loosely associated with the membrane at a K_D in the range of 190nM (Jensen et al, 2011), lipoproteins stay anchored with a K_D of .54nM (Hu et al, 2015). This ensures that the protein remains at the membrane. Much of the work performed on lipoprotein modification, secretion and localization was done in *E. coli* and the pathways described here are from *E. coli* unless otherwise stated.

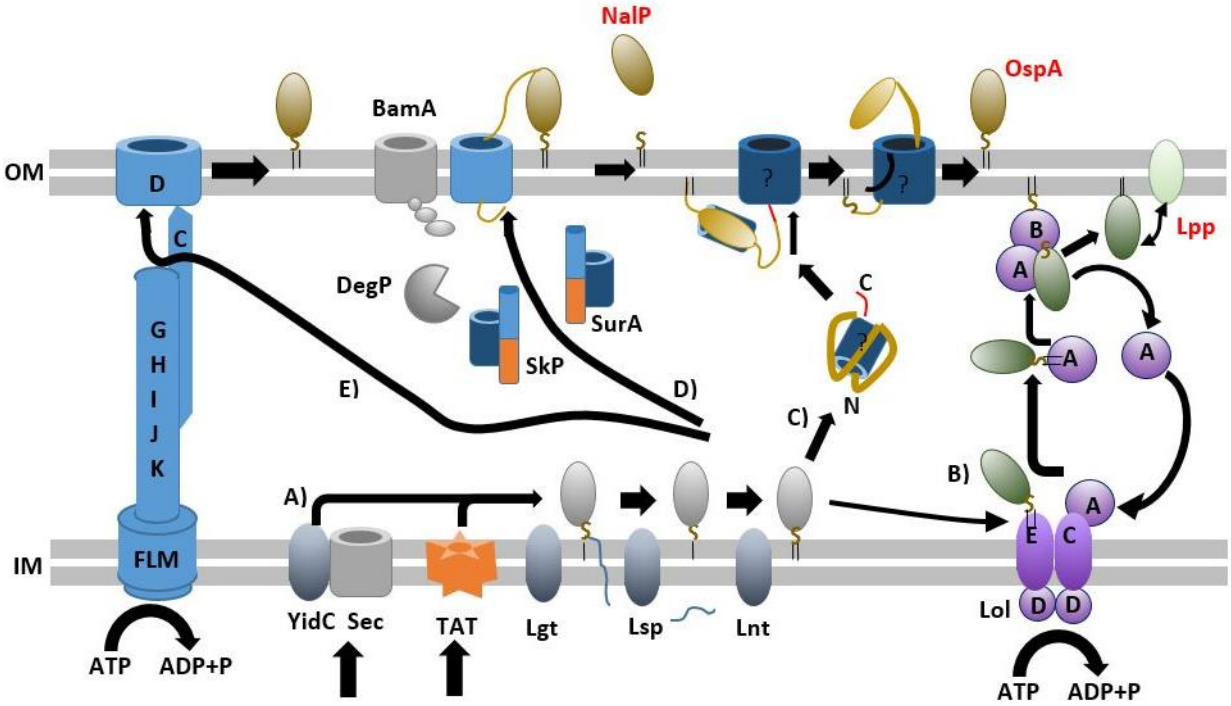

Figure 5. Lipoprotein Secretion Machinery. **A)** Upon export of the prolipoprotein via either the Sec or Tat machinery, the proliprotein will be lipidated, cleaved, and further lipidated by the lipidation machinery proteins Lgt, Lsp, and Lnt respectively. The Secretory complex (Sec) or Twin Arginine Transporter (TAT) are responsible for transporting proteins with a signal peptide and placing them in the innermembrane with the help of YidC. Once lipidated by Lnt, the newly completed lipoprotein must be properly localized. **B)** The Lol pathway is responsible for removing lipoproteins from the inner membrane to the outer membrane. **D)** The type II and **E)** type V secretion system directly translocates lipoproteins to the outer surface. The *N. gonorrhea* protein NalP is secreted through the type V secretion system and will autolytically cleave to become a part of the surrounding environment. The putative spirochete secretion system is shown in **C)** with the potential to interact with the Lol pathway (modified from Zückert, 2014)

4.2 Secretion, Cleavage and Lipidation (Fig. 5)

Upon recognition of an export signal at the N-terminus of the nascent lipoprotein, a cytoplasmic chaperone will transport the newly synthesized prolipoprotein to the inner membrane. Once transferred to either the Sec or Tat machinery, which will export newly synthesized proteins and insert them into the cytosolic membrane (Froderberg et al, 2004, De Buck et al, 2004). As the protein is being exported, the lipidation machinery will recognize a 5-amino-acid motif (called a lipobox) at the C terminus of the export signal peptide (Wu, 1996). The lipobox includes a conserved cysteine that will be modified with phosphatidylglycerol fatty acid group (Wu et al, 1982). The identity of the amino acids that constitute the lipobox can vary. For *E. coli* the consensus lipobox is comprised of LLAGC while for *Borrelia* the consensus sequence is LLIAC (Haake, 2000). There is some variation in the first four amino acids of the lipobox, and the congruency of the sequence is greater in Gram negative and Gram positive bacteria than it is for spirochetes.

Upon export and insertion of the prolipoprotein by either the Sec YEG or Tat pathway, a diacylglyceryl molecule is attached via the prolipoprotein diacylglyceryl transferase (Lgt) to the soon to be N-terminal cysteine (Tokunaga et al, 1982). Lgt has 7 transmembrane domains and performs its enzymatic activity within the cytoplasmic membrane (Pailler et al, 2012). Phosphatidylglycerol is the substrate of choice for this modification while phosphotidylethanolamine, phosphotidyl serine and cardiolipin are excluded from this reaction. The interaction between Lgt, the target lipoprotein, and phosphotidylglycerol has yet to be determined, but amino acids essential for Lgt function are located within the transmembrane domains (Pailler et al, 2012). Regarding the acyl chains, mass-spectrometry data of modified lipoproteins have suggested that there is little to no selection concerning which acyl chain is

attached to the prolipoprotein, and the lipid moiety percentages are based on the fatty acid composition ratios of the species of bacteria in question (Buddelmeijer, 2015). Since *Borrelia* acquires its fatty acids from the environment, logically the acyl chain modification would be dependent on the environment, although this has not been experimentally shown.

Upon the addition of the diacylglyceryl moiety to the sulfhydryl group of the +1 cysteine, the export signal peptide is removed before the addition of another fatty acid to the amino nitrogen the "+1" cysteine (Tokunaga et al, 1984). This is performed by the prolipoprotein signal peptidase (Lsp) (Tokunaga, Loranger, Wu, 1983). Lsp is a conserved aspartic protease that can be transferred among bacterial species readily and still function (Pragai et al, 1997). It has 4 transmembrane segments and 6 required active site amino acid residues (Munoa et al, 1991). Lsp recognizes the signal peptide with the attached thio-acyl group, and as this thiolipid is required for the enzyme to recognize and bind the target prolipoprotein, the modification of the lipoprotein is ensured to proceed in a stepwise manner (Tokunaga et al, 1983). Cleavage of the signal peptide can lead to other modifications of the newly released short peptide chain which can play a role in cell signaling in some bacteria (Cook and Federle, 2014). However, the usual endpoint for the signal peptide is degradation by a signal peptide peptidase (Pecaud, 1982). The "+1" cysteine residue is now the N-terminus of the apolipoprotein.

Upon cleavage of the signal peptide the apolipoprotein will undergo further modification with two additional fatty acid chains. These acyl chains are attached to the amine nitrogen of the "+1" cysteine of the apolipoprotein (Gupta and Wu, 1991). This reaction is catalyzed by the apolipoprotein N-acetyltransferase (Lnt). In vitro, Lnt prefers to use phosphotidylethanolamine with 16-18 carbon acyl chains, but it can utilize other phospholipids with small polar head groups (Hillmann et al, 2011). Lnt first removes the excess acyl chain from its phospholipid donor by

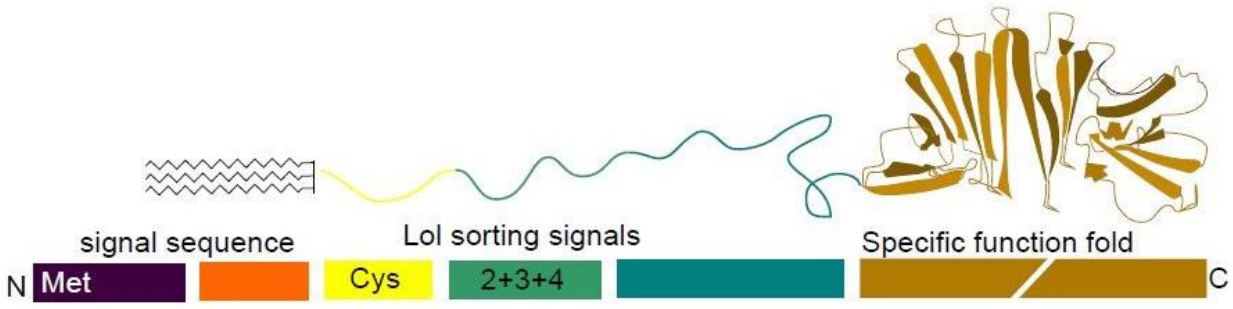

Figure 6. Lipoprotein Structure. As the nascent prolipoprotein is being synthesized in the cytoplasm the N-terminal signal sequence (purple and orange) will interact with a chaperone that will deliver the lipoprotein to the Sec or Tat pathway. Moving towards the C-terminus of the protein, the lipobox (orange and yellow) will signal the interaction between the +1 cysteine and lipid adding machinery of Lgt, Lsp and Lnt before the fully mature lipoprotein is localized to its final destination. A specific amino acid sequence following the +1 cysteine is responsible for the proper localization of the lipoprotein (green). Once properly located, the tether, a small unfolded region which includes the localization signals, separates the lipoprotein from the lipid bi-layer (green and blue). The functional domain (gold) of the lipoprotein is found at the C terminal end, and can be mostly unstructured or highly structured (modified from Zückert, 2014).

forming a thioester substitution intermediate with the acyl chain to be transferred. This creates an acyl-enzyme intermediate that will attach the acyl chain to the +1 cysteine to form a triacylated protein (Buddelmeijer and Young, 2010). For most bacteria the triacylated form is the mature lipoprotein. However, *Borrelia* seems to have a unique system in which one of the acyl chains from the thioglyceryl moiety is replaced with an acetyl group. This leaves a lipoprotein with only two fatty acid chains and an acetyl moiety as the mature form of lipoprotein (Beerman, 2000).

4.3. Lipoprotein Sorting (Fig. 5)

Once the lipoprotein has been fully modified and matured, it must now move to its final location. In *E. coli* the vast majority of lipoproteins are found on the inner leaflet of the outer membrane (Tokuda, 2007). In Gram-negative bacteria, lipoproteins are quite frequently used for membrane stability to balance the bulky lipopolysaccharide on the outer surface. The most abundant lipoprotein in *E. coli*, Lpp, is responsible for maintaining membrane stability and homeostasis (Suzuki et al, 1978). In more recent work Lpp has been shown to localize to two different cellular compartments dependent on binding of peptidoglycan. This limits Lpp's ability to translocate to the outer membrane (Cowles et al, 2011). For bacteria without LPS, like *Borrelia*, the ratio of inner membrane:inner leaflet of the outer membrane:outer leaflet of the outer membrane are much different. Data from our own lab has place the ratios at 30%:5%:65% (Dowdell et al, 2017).

If the mature lipoprotein has a final localization to the inner membrane, no further action is required for correct localization. However, since the majority of the lipoproteins by abundance and by number are in the inner leaflet of the outer membrane, outer membrane lipoproteins must be released from inner membrane (Tokuda, 2007). The Lipoprotein outer membrane localization (Lol) pathway is responsible for moving lipoproteins from the inner membrane to the outer membrane and is essential for survival of the bacterial cell. Discovered in 1995 and elucidated in

the following years, the Lol pathway is an important feature of the periplasmic space (Matsuyama et al, 1995). Composed of 5 proteins, the Lol pathway includes proteins in the cytoplasm, inner membrane, periplasm, as well as the inner leaflet of the outer membrane. The LolCDE complex is the ATP driven launching pad for LolA, which is the shuttle for the lipoprotein. LolB acts as a dock for the LolA-lipoprotein complex and inserts the lipoprotein into the outer membrane. Detailed pathway functions have been teased from the *E. coli* LolA pathway transporting Lpp and Pal. Upon acylation by Lnt, the LolCDE complex binds the mature lipoprotein (Yakushi et al, 2000). First interacting with LolE, the lipoprotein is transferred from Lnt to LolE. This causes conformational changes in LolD which in turn binds ATP (Mizutani et al, 2013). Since removing lipids from the lipid bilayer is energetically unfavorable due to hydrophobic forces, cytoplasmic LolD provides the energy to overcome this unfavorable reaction. By converting ATP to ADP, LolD allows for the removal of the lipoprotein from the inner membrane and transfer of the lipoprotein from LolE to an unloaded LolC:LolA complex. Conformational changes caused by LolA's binding to the lipoprotein releases LolA:lipoprotein from the LolCDE complex (Okuda and Tokuda, 2009). LolA then acts as the periplasmic chaperone that transports the lipoprotein across the periplasm to the outer membrane (Yakushi et al, 1998). LolB, already present on the inner surface of the outer membrane, receives the lipoprotein and inserts it into the outer membrane (Matsuyama et al, 1997). Both LolA and LolB bind the lipid moieties of the lipoproteins using a hydrophobic pocket that allows for interaction with the full range of lipoproteins. It has been suggested that due to the greater binding affinity of LolB than LolA to lipoproteins, the reaction flows from inner membrane to outer membrane, even though transfer from LolB to LolA is theoretically possible (Taniguchi et al, 2005).

4.3.1 Sorting Signals

Upon cleavage of the signal peptide, the now mature lipoprotein contains another signaling mechanism for the sorting of the lipoproteins. In *E. coli*, the +2 position is the determining amino acid localization in most cases. Aspartic acid in the +2 position retains the lipoprotein in the inner membrane while other amino acids such as serine in the +2 position allow for the movement of lipoproteins to the outer membrane (Yamaguchi et al, 1988). There is growing evidence that the +3 position influences the localization of lipoproteins in *E. coli*, and the +3 and +4 positions have been implicated in other gram negative bacteria as important residues for proper localization. (Lewenza, 1998).

In the case of *B. burgdorferi* and some others, the Lol pathway is incomplete as there is no detectable LolB homolog (Okuda and Tokuda, 2011) (Zuckert, 2014). The sorting rules are also different in *B. burgdoferi* as many different amino acid residues are found in the +2 position of outer surface proteins (Schulze et al, 2010). Also missing from *Borrelia* are key components of the type II and type V secretion systems that place proteins on the outer surface of some Gram negative bacteria (Fraser et al, 1997) (Zückert, 2014). Work done in our lab has shown that mutations in the intrinsically disordered region between the lipidated cysteine and the folded protein (called the "tether") can cause outer surface lipoproteins to mislocalize to the periplasmic face of the outer membrane (Kumru et al, 2011). Although the mechanism remains unclear, our hypothesis is that these mutants cause premature folding in the periplasm. This may possibly be due to lack of interaction with a putative periplasmic holding chaperone and the hypothesized lipoprotein flippase complex in the outer membrane (Schulze and Zuckert, 2006) (Zückert 2014).

5. Overall Goal

The overall goal of this thesis project was to establish either a biochemical or genetic screen to identify key lipoprotein secretion pathway components in *Borrelia burgdorferi*. The project also further explored potential spirochetal lipoprotein sorting signals.

6. Approach (Fig. 7)

To identify any sorting signals responsible for localizing lipoproteins to their respective locations, our lab has undertaken a series of mutational analyses of the intrinsically disordered "tether" region that lays between the lipidated cysteine anchor and the structured, functional fold of each lipoprotein (Figure 6). One potential function of the tether may be to provide the necessary distance between functional protein domains and the membrane (Zuckert, 2014), situating the protein optimally within the surface proteome. The tether regions for lipoproteins vary greatly in amino acid length. OspA has a 12-amino-acid tether while CspA has a 33-amino-acid tether. With 170 amino acids, the longest unstructured tether in a *B. burgdorferi* lipoprotein belongs to BBA66 (Brangulis et al, 2014). The functions of these lipoproteins provide some insight that function may dictate form. OspA is both immunoprotective against antibodies and binds to a protein in the tick midgut which may favor a close, tight binding. CspA, however is an important anti-immune response protein (Hammerschmidt et al, 2014). Because of the randomly activating nature of the Alternative Complement Pathway, CspA may act as an extended shield, using factor H to prevent the further action of C3b in forming the membrane attack complex. Thus, the more extended the shield (the longer the tether), the more protection may be offered. The function of BBA66 is undetermined but it is important for transmission to the host (Patton et al, 2013). In previous work performed by our lab, we used deletion and mutagenesis of this tether region to create subsurface variants of OspA.

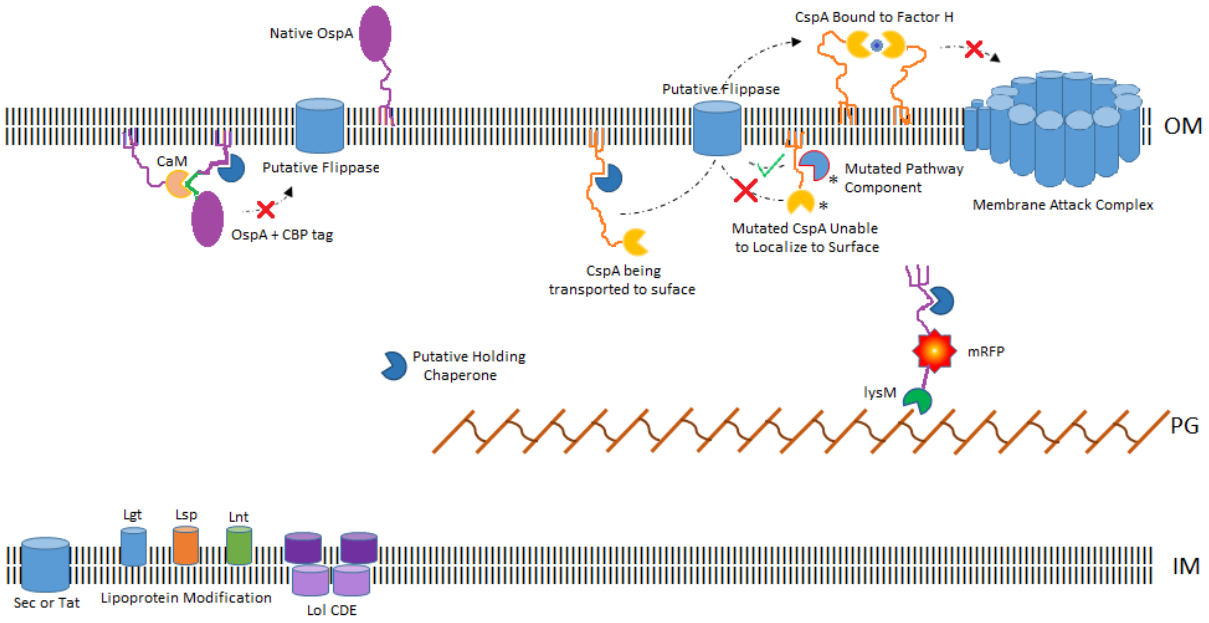

Figure 7. Approach. Two strategies taken to determine the periplasmic components involved in localizing lipoproteins, substrate trapping and mutation. In substrate trapping we attempt to use binding partners to physically hold the lipoprotein in the periplasm. This increases the number of lipoproteins bound to their interacting partners and allows for crosslinking methods to be employed. One method of substrate trapping attempted was the use of a lipoprotein calmodulin binding to a OspA:CBP fusion stopping the secretion at the inner surface of the outer membrane. Another method involves the interaction between LysM and peptidoglycan (PG). Using the native OspA tether fused to an RFP marker and a native LysM domain, the protein should be located in the periplasm. CspA is a serum protective lipoprotein that binds to Factor H and prevents lysis by complements Membrane Attack Complex. If a subsurface CspA could be created, the membrane would become susceptible to lysis by human serum. A suppressor screen would identify any mutations that revert CspA to the surface.

6.1. CspA Mutational Analysis

As mentioned above, CspA is an important gene in the life cycle of *Borrelia* because it offers protection from complement. While understanding which amino acid combinations in the tether produce surface, subsurface and inner membrane mutants was one of the secondary goals of this study, the creation of a subsurface CspA through tether mutation would allow for a suppressor screen to be run. Because a subsurface CspA mutant would be vulnerable to lysis by complement, *Borrelia* would be unable to survive in a complement rich media. Any mutation that causes restoration of the mutant lipoprotein's localization to the surface (i.e., any additional mutation that suppresses the original mutant phenotype) would confer protection to the bacteria and allow them to grow in the presence of complement. Reversion can occur in two ways: a compensatory mutation in CspA that allows for CspA to pass to the surface, or a mutation in any lipoprotein secretion pathway protein that causes the subsurface mutant CspA to be exported to the surface in spite of its subsurface localization signal. Reversion mutants of CspA can reveal more about the amino acid sequence signals required for export, and mutations in other proteins lead to a greater understanding of the export pathway components. Creation of the subsurface mutants was attempted in a two-fold approach. The primary approach was to isolate the necessary component of the export signal within the CspA tether through mutational analysis. A secondary approach was to fuse CspA with a previously known subsurface lipoprotein tether.

6.2. Periplasmic Trapping of Lipoproteins

While we have a full complement of his-tagged lipoproteins as well as antibodies to a wide array of lipoproteins that localize to the three residencies of lipoproteins, co-immunoprecipitation of these proteins has not resulted in the identification of lipoprotein pathway components by MudPIT mass spectrometry. Processing and transport of these proteins appears to be fairly quick, and

therefore the majority of surface and subsurface lipoproteins are in their final locations at steady state. This means the process must be slowed down or stalled, similar to work done with VSV viral infection in Eukaryotic systems to study protein export and localization in the Golgi. (Miranov et al, 2001). We attempted to physically stall protein movement through the mutational addition of known protein binding domains of calmodulin and LysM.

6.2.1. Calmodulin

Calmodulin is a small, highly conserved eukaryotic protein that changes conformation in the presence of Ca^{2+}, causing the protein to interact with other proteins via a calmodulin binding domain (CBD). This conditional binding can be exploited, e.g., in protein purification protocols, by using a high-affinity recombinant calmodulin binding peptide (CBP) (Egorov et al, 2004). Our lab has used another feature of calmodulin to show that the unfolding of the proteins in necessary for secretion to the outer surface by manipulating Ca^{2+} levels in the media (Chen and Zuckert, 2011). By inserting a CBP onto a peptide that normally localizes to the bacterial surface and introducing a calmodulin that localizes to the periplasm we hypothesized that the lipoprotein:CBP fusion would be stalled in the periplasm with any associated transport proteins. In these experiments, we used OspA as a model surface lipoprotein with both a C-terminal and N-terminal CBP tag.

6.2.2. LysM

The LysM protein domain is a GlcNAc binding domain that is used by multiple proteins in all kingdoms. Peptidoglycan contains long chains of GlcNAc, and LysM domains are frequently used to anchor proteins to the bacterial cell wall with some proteins containing multiple LysM domains (Mesnage et al, 2014). Work done in *S. aureus* has shown that the LysM domain can be used to

hinder the progression of protein excretion when attached to proteins (Ebner et al, 2015). In this study we used multiple repeating LysM domains in an attempt to anchor a wild type OspA tether:mRFP fusion to the periplasmic peptidoglycan.

7. Results

7.1. Generating a Subsurface CspA Mutant

A series of CspA deletion mutants were created using overlap extension PCR to find the minimum amino acid tether sequence required to locate CspA to the surface. Cysteine 25 represents the N-terminus of the mature CspA lipoprotein and the end of the lipobox. Data from previous experiments showed that both a deletion of amino acids 26-45 and 46-63 amino acids did not prevent surface localization in *B. burgdorferi*. That experiment was repeated here and is shown in Figure 8. Further deletions, in 4 amino acid increments, were made to extend the deleted tether portions towards the N-terminus, continuing from the available Δ46-63 mutant (see Table 2 for oligonucleotide primers). The mutants' localizations were tested using a standard protease accessibility assay, incubating with Proteinase K at a final concentration of 200 µg/mL for an hour at room temperature. Proteinase K will degrade any accessible proteins at the surface of the bacterial cell membrane. While this can cause membrane instability, great care is taken to ensure that the membrane remains uncompromised and does not allow Proteinase K access to the periplasm or the cytoplasm. These methods include gentle buffers and slow centrifugation speeds. Whole cell lysates were separated on a SDS PAGE, normalized for total protein amount, and analyzed by Coomassie staining and western blotting with protein-specific antibodies.

After analysis, all CspA tether deletions remained localized to the surface, including the amino acid 30-63 deletion which did not bind the available monoclonal anti-CspA antibodies. This Δ30-

63 mutant was clearly shown in the Coomassie stain as sensitive to proteinase K (Figure 8), but the mapped CspA epitope lay just past the tether region (Kraicsy et al, 2004) which could be important for proper folding of the protein. To get around the limitation in analyzing this Δ30-63 mutant, a poly-histidine tag was placed on the C-terminus of the mutant protein to help determine its localization. Yet, the protein remained undetectable by a His-probe or by an anti-poly-histidine antibody (Figure 8). One thought is that a peptidase could be responsible for cleaving the his-tag from the C-terminus.

In previous studies, we generated subsurface tether mutants of the surface lipoprotein OspA (Schulze and Zuckert, 2010). Thus, in an alternative approach, we fused a wild type surface OspA tether and the tether of a subsurface mutant OspA to the functional fold of CspA. Neither the wild type or mutant OspA tether:CspA fusions were detectable through CspA antibodies or through histidine antibodies after the addition of a C-terminal histidine tag (Figure 8). Yet, Coomassie staining showed that a band matching the size of both the OspA tether:CspA fusions were degraded by proteinase K. Thus, we surmise that the proteins were, in fact, located to the surface.

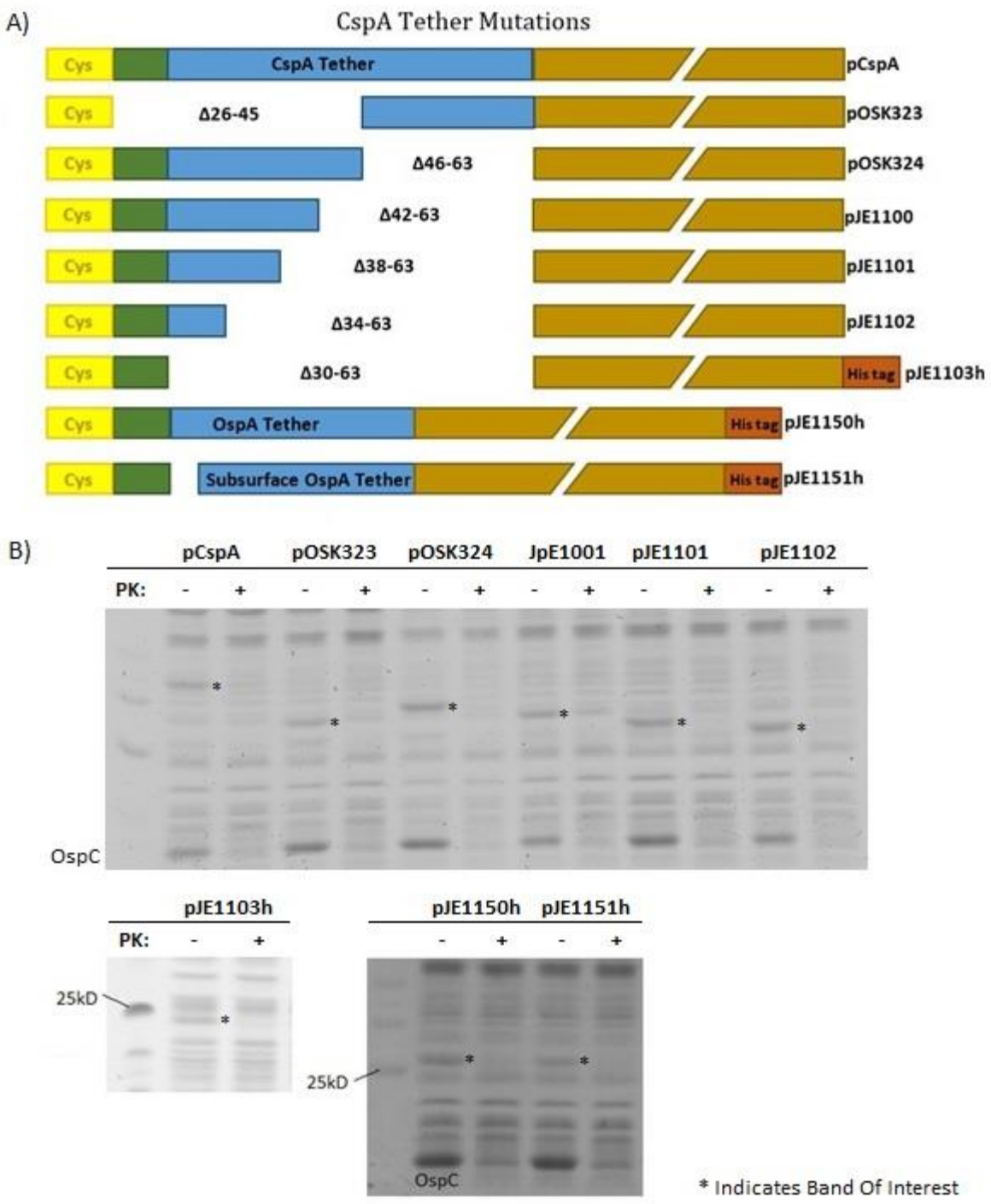

Figure 8. CspA Mutations. **A)** CspA tether deletions, indicated by Δ#-#, and mutations, described below, were made to attempt to create a subsurface CspA to be used in a suppressor screen. The mutations were made in a systematic manner to determine the minimum required tether for correct localization. In addition to deletions, a subsurface mutant tether from OspA ΔV21 (Schulze et al, 2010) as well as a WT OspA tether control were added as a failsafe to ensure a subsurface localization. **B)** Proteinase K (PK) will digest only surface proteins of cells with intact membranes. Wild type CspA is found on the surface of *Borrelia* during the mammalian phase, and the corresponding band disappears

after an hour of treatment with proteinase K at 200µg/mL indicating that the protein is on the surface. After PK treatment, no tether deletions or mutations were found to be subsurface either by western blot and antibody staining or by Coomassie staining (shown) including the OspA subsurface tether.

7.2. OspA Fusions to Calmodulin-Binding Peptide

Using Gibson assembly (New England Biolabs), a calmodulin binding peptide (CBP) was introduced to OspA. Two CBP:OspA fusions were made, one at the OspA C-terminus and one at the N-terminal end between the tether and functional fold. Upon proteinase K treatment both C-terminal and N-terminal fusions were determined to be localized at the surface of B31e2, a high passage strain of *B. burgdorferi* (Figure 9a). With the confirmation of normal localization for both the C-terminal and N-terminal CBP fusions, we took two approaches to introduce calmodulin into the OspA:CBP system. First, the recombinant plasmid already expressing the OspA:CBP fusion was modified by adding a module expressing an outer membrane-anchored, periplasmic calmodulin under a tetracycline-responsive promoter (Post, Whetstine et al, 2009). The resulting plasmid was used to transform E. coli, and the correct sequence was confirmed by single-pass sequencing (ACTG, Inc.) Induction of expression of calmodulin by addition of anhydrotetracycline in *E. coli* was also confirmed (Figure 9b). Yet, no transformants of high passage *B. burgdorferi* strain B31 clones B31e2 or B313 were ever recovered. Since the vector carrying both calmodulin and the OspA:CBP fusions were approximately 10kb in size, which may affect the transformation efficiency for *Borrelia*, we decided to take a two-plasmid approach. In this second approach, a suicide plasmid (Figure 9b), unable to replicate in *B. burgdorferi*, was designed to place the tetracycline-inducible subsurface calmodulin module on the main chromosome *via* homologous recombination. The suicide plasmid was constructed without any replication or growth defects in *E. coli.* In addition to electroporation with the circular suicide plasmid, the plasmid was cleaved with a single cutting restriction enzyme (Acc1) to create a non-supercoiled linear piece of DNA available for homologous recombination in *B. burgdorferi*.

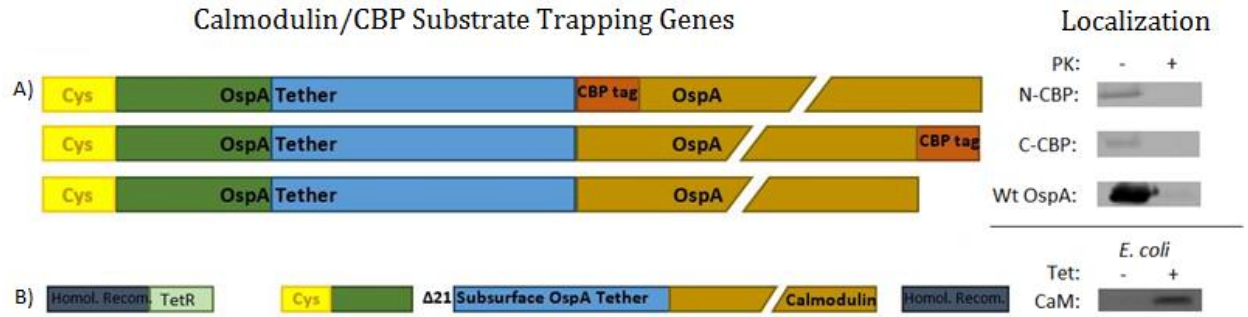

Figure 9. OspA Mutations. A) Creation of OspA mutants to include a short peptide binding site for calmodulin. The addition of the CBP at either the N-terminus or C-terminus had no effect on the lipoproteins ability to localize to the surface as shown by proteinase K treatment for 1 hour. **B)** A suicide plasmid containing an anhydrotetracycline (TetR) inducible calmodulin (CaM) and an CBP:OspA fusion was able to vary expression of CaM in the presence of anhydrotetracycline in *E. coli*, but no clones either via single plasmid (with the CBP OspA) or homologous recombination (TetR:CaM only) were ever recovered in high passage *Borrelia* strains B313 or B31e2.

Neither the linear nor the circular forms of the suicide plasmid yielded transformants in *B. burgdorferi* strains B31e2 and B313.

7.3. Fusion of a *B. burgdorferi* LysM Domain to a OspA28:mRFP Fusion

The LysM domain belonging to the *B. burgdorferi* gene BB0323 was fused to a red-fluorescent lipoprotein that localizes to the bacterial surface using the wild-type OspA tether (OspA28:mRFPΔ4; Schulze and Zuckert, 2010). The OspA:mRFP:LysM fusion was placed under the control of the constitutive *B. burgdorferi flaB* promoter. We hypothesized that upon addition of the LysM domain to the C-terminal end of the fluorescent lipoprotein, the fusion protein would bind periplasmic peptidoglycan and, thus, be blocked from localizing to the surface. Although the *E. coli* transformants did fluoresce red (Figure 10D), the bacteria had noticeable divisional defects and their growth rate was significantly lower (Figure 10ABD). Additionally, DNA extraction yielded poor amounts of low quality plasmid. Thus, upon electroporation, no *Borrelia* transformants of clone B313 were recovered either due to poor yield or toxicity of the plasmid.

8. Discussion

Results from our previous studies have suggested that the default pathway for lipoproteins in *Borrelia* leads them to the surface. This means that a form of retaining signal is required to prevent movement across the periplasm and movement to the surface (Schulze and Zuckert, 2006). One can imagine any number of scenarios in which lipoproteins interact with transport chaperones, folding chaperones, flippases, proteases, and modification enzymes that all play some role in moving lipoproteins to their predetermined location. Due to the fact that the majority of lipoprotein species are already in their final location (*i.e.*, not bound by localization machinery), whether that be on the outer surface of the inner membrane, the inner leaflet of the outer membrane or on the

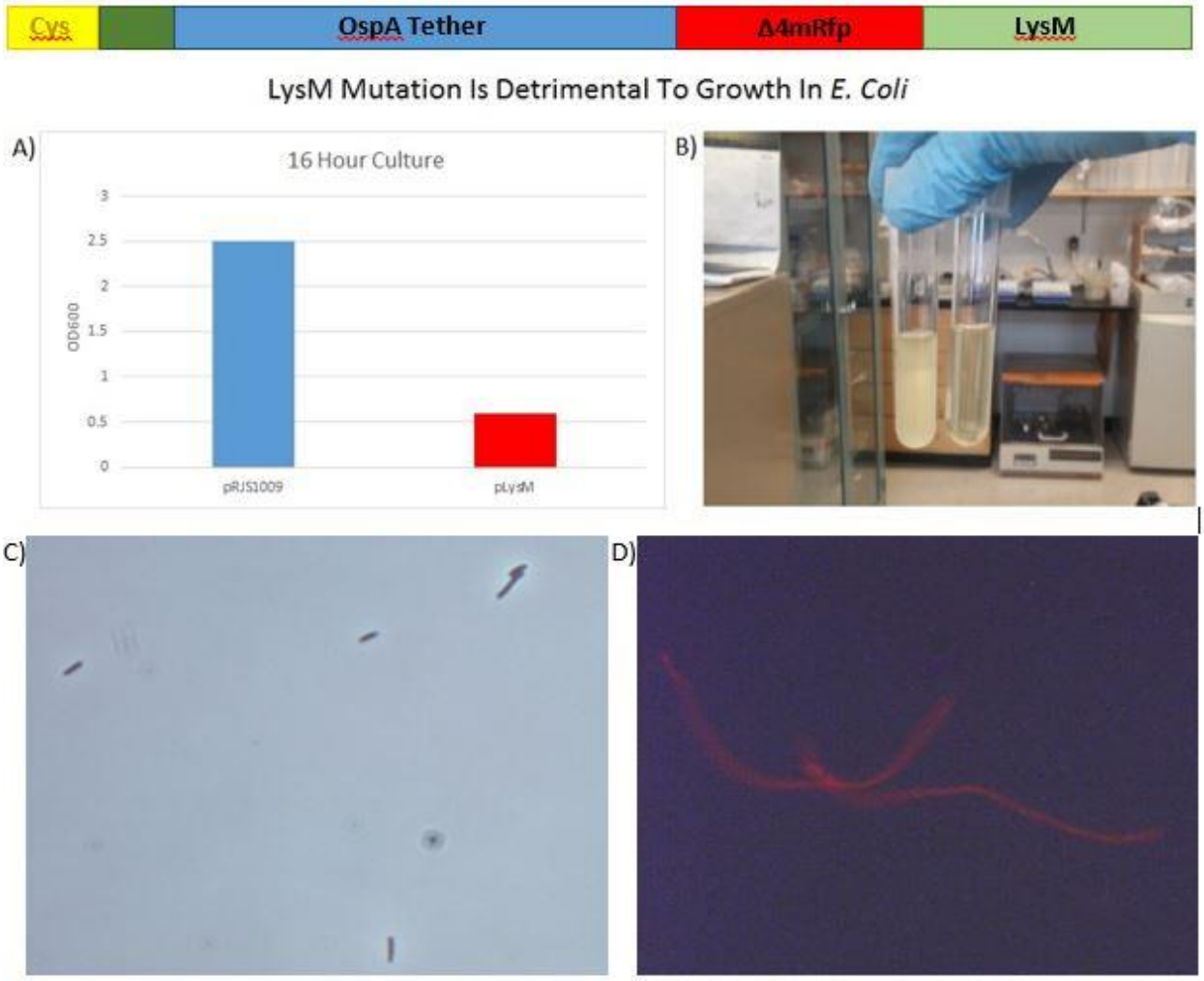

Figure 9. LysM Toxicity. A surface localizing mRFP fused to LysM was created to bind a lipoprotein to peptidoglycan (gene schematic shown above, colors as in Fig. 6). **A) and B)** The plasmid containing the LysM fusion proved to inhibit growth in *E. coli* upon transformation in optical density at 600nm (**A)**) as well as visual inspection (**B)**) compared to the control vector pRJS1009. No positive tranformants were ever recovered in high passage *Borrelia* strains B313 or B31e2. **C)** While a vector with just mRfp and no LysM domain caused no growth defect, **D)** *E. coli* containing the LysM domain with mRfp showed significant elongation and filimentation. The culture in **C)** was diluted 10x to be a similar concentration as **D)**.

surface of *Borrelia*, it is difficult to study the system with the limited number of proteins in the state of transport (i.e., bound by the localization machinery). Earlier crosslinking analyses in our lab using lipoproteins as baits have not identified the pathway components. This study's attempts to interfere with transport of lipoproteins have proven to be deleterious, at least under the conditions tested. While surface localization may be the default pathway for lipoproteins in *Borrelia*, there continues to be a need for a biochemical interaction with the components moving through the pathway. In Gram-negative bacteria, such as *E. coli* or *Pseudomonas*, there is clear signaling in the +2 through +4 amino acids that determine localization, with little variance. In *Borrelia*, the +2 through +4 amino acids are highly variable (Setubal et al, 2006). As this study has suggested, the requirement of the CspA tether to localize to the surface is less than its requirement for it to fold correctly. Additionally, a mutant tether that causes OspA to mis-localize to the inner leaflet of the outer membrane does not restrict CspA to the same cellular compartment. This, along with previous data showing that amino acids 46-63 can act as a functional tether for CspA, suggests that other mechanisms besides tether recognition may be responsible for the transport of CspA across the periplasm and outer membrane. Our lab has previously shown that a folded conformation in calmodulin can prohibit translocation across the outer membrane (Chen and Zuckert, 2011). In addition, our data has suggested that initiation of movement across the outer membrane occurs via the C-terminus of surface lipoproteins. This suggests that the C-terminus could be important for localization, in addition to the signaling performed at the N-terminal end of the mature lipoprotein.

This study limited the necessary requirements for proper localization of CspA to the +2 through +5 amino acids. Additionally, this research shows that, in the case of CspA and the OspA subsurface mutants, the localization of one tether does not determine the localization of the same

tether attached to another protein (Figure 8). While many mutants capable of being trapped within the periplasm were made, introduction of the trapping mechanism proved deleterious in two separate systems. This shows that functioning lipoprotein transport is a vital part of *Borrelia burgdorferi* (Figures 9 and 10).

9. Future Studies

While this study was successful in limiting the minimally required tether length for export of CspA to the surface to the first four amino acids following the +1 cysteine, no mutations of the first four amino acids of the CspA tether have been placed into *Borrelia*. A mutational analysis of these amino acids could prove to be informative. Previously, deletion of the whole tether seemed to cause degradation of the CspA gene, or at least ablates detection with an antibody (unpublished data). Interestingly, the tethers that produces a surface or subsurface OspA prevent detection by CspA monoclonal antibodies when fused to CspA (likely due to epitope obfuscation) and do not prevent movement of CspA to the surface. This suggests either multiple mechanisms of transport are at play, or some signal is hidden in the combination of the OspA tether and CspA functional fold. Clearly, more research is required to approach the problem of what is necessary and required for lipoprotein sorting signals and their proper localization. Alongside this research, our lab has recently published a paper cataloging the surface localization of all the lipoproteins in *B. burgdorferi* (Dowdell et al, 2017), and no strong consensus sequence was found. Since all of the sequences of these proteins are known, hypotheses regarding consensus sequences can be better informed in future studies. While we were currently unable to make a subsurface CspA mutant, the elucidative power of such a mutant prevents us from immediately abandoning the attempt to create it. From the data provided in the paper above, I hypothesize a mutation of the +3 amino acid residue to serine or threonine would create a subsurface mutant. Additionally, because the naturally

occurring subsurface lipoprotein tether sequences are now known, I would perform a tether swap with these tethers in a similar manner to what was done with the OspA subsurface mutant. If we are successful in making a subsurface mutant CspA, the suppressor screen described above would be the next logical step.

On the subject of trapping the lipoprotein within the outer membrane, an ever present concern is the possibility of the trapping conditions being toxic to the bacteria. Calmodulin and CBP showed no apparent toxicity in *E. coli*. Since the CaM-CBP system of entrapment is completely foreign to *Borrelia*, the risk of toxicity is low. Nevertheless, it is concerning that no viable *Borrelia* were recovered upon transformation of the suicide plasmid without a CBP protein present. Creation of the tet-inducible calmodulin suicide plasmid was one attempt at mitigating such toxicity during growth. After sufficient number of cells have grown, we would be able to study the effects of the entrapment using the inducible promoter. Also, because we know that a subsurface calmodulin is negligibly toxic (Chen and Zuckert, 2011), a redesign of the suicide plasmid's homologous recombination site may be called fo to bring this system to a place where calmodulin can be introduced into *Borrelia's* genome. This would allow for the sorting of the OspA:CBP fusion to be studied without risk of toxicity during the transformation process. When it comes to LysM binding peptidoglycan, the toxicity of the system readily displays itself in *E. coli*. The introduction of an inducible promoter for the LysM:OspA:mRrp gene is the next logical step in addressing the toxic side effects of this native protein binding system.

10. Materials and Methods

Bacterial Strains and Culture Conditions.

The *E. coli* Top10 strain (Invitrogen) was the strain used for all recombinant DNA construction and recovery. *E. coli* strains were grown in Luria Bertani (LB) broth/agar (Difco). Frozen stocks of *E. coli* clones were made using 15% glycerol (v/v) into LB broth and kept at -80°C. The *Borrelia burgdorferi* strains B31-e2 and B313 are clones of the type strain B31 and were used for the expression of recombinant plasmids. B31-e2 expresses the linear plasmid lp54 which contains the genes encoding OspA and CspA. B313 lacks lp54. Both *Borrelia* strains were grown in BSK-II liquid/solid media. Frozen stocks of *B. burgdorferi* clones were made using 10% DMSO (v/v) into 1x BSK-II complete.

SDS PAGE and Immunostaining.

Protein samples were separated using a 10% polyacrylamide gel. Initial protein visualization was performed using Coomassie blue staining. If needed protein levels were balanced through visual inspection before nitrocellulose transfer and immunostaining. Transfer of proteins from polyacrylamide gels to nitrocellulose membranes was completed using the Transblot-SD Semi-dry Transfer Cell (BioRad). After blocking with either a 5% milk solution or a 2.5% BSA solution, primary antibodies in blocking solution were applied to the nitrocellulose membrane for one hour at room temperature (RT) or overnight at 4° C. Membranes were then washed with 1x TBS-T, .05% Tween-20. After washing, secondary antibodies conjugated with alkaline phosphatase in blocking solution were added to the membranes and allowed to incubate for 1 hour at RT. The membranes were then washed with TBS, incubated for 10 minutes at RT with Lumiphos WB (Thermo Scientific) and visualized in 30 second exposures up to 10 minutes.

PCR, Agarose Gel Electrophoresis, and Sequencing.

Polymerase chain reactions were performed with either Taq polymerase or Q5 high fidelity polymerase according manufacturer recommended protocols. Overlap extension primers were design with a melting temperature (Tm) of 55° C. See Table 2 for individual primer molecules used. TAQ polymerase was use in 1x ThermoPol buffer, 0.2 mM primers, 0.2 mM dNTPs and ~1ng/mL template. Q5 polymerase was used in 1x Q5 buffer, 0.5 mM primers, 0.3mM dNTPs, and ~1ng/uL template. Agarose gels were made between 0.75% and 1.25% (agarose type) in 1x TAE buffer to separate DNA by size. DNA samples were placed in the gel matrix and subjected to a 100V, 400 mA current for 30 to 45 minutes depending on predicted DNA size. Sample were visualized using ethidium bromide staining. Sequencing results were obtained by Eton Biosciences, Inc. and ACTG Inc.

Plasmid Construction and Lipoprotein Mutations.

CspA tether mutations were made in two separate PCR reactions. The first reaction was to create an overlap as well as add a BamH1 site beyond the 5' end of the promoter region and HindIII site at the 3' end of gene. The second reaction merged the two ends of the gene. Upon restriction these mutated CspA genes were ligated into the pKSSF1 shuttle vector and were confirmed by sequencing. See the primer list and plasmid list for additional construction details (Table 1 and 2). For his-tag addition an elongated 3' primer with a poly-glycine buffer region, a 6 histidine tag with a stop codon and a HindIII restriction site were used with the 5' BamH1 primer. After PCR amplification, an additional amplification step with Taq polymerase was used to create a 3' adenine overhang that was inserted in to the TOPO TA vector. Upon insertion and amplification on *E. coli* the gene was restricted and ligated into pKSSF1 and confirmed by sequencing. OspA:CBP were created using Gibson assembly. Primers were designed to create overlaps with a Tm of 48°C

between the 3 segments to create a pBSV2 backbone, CBP tag, and OspA under the Pflab promoter. A subsurface CaM under a tetracycline inducible promoter was then added to these genes as well as a wild type OspA control. These genes were confirmed by sequencing. The OspA:mRfp:LysM hybrid was made using two segments. A surface localizing (native OspA) tether was fused to mRfp under control of the Pflab promoter, and a 5' HindIII site was fused with a native 6 repeating LysM domain structure from the chromosomal *Borrelia* gene BB0323 containing with the primer adding a 3' BamH1 site. This gene was placed in pBSV2.2 which contains a gentamycin resistance cassette instead of kanamycin resistance found on pBSV2.

Proteinase K Assay.

12 ml Borrelia cultures were grown to a concentration of 1.0 x 10^8 cells/ml. Cultures were split in half and centrifuged at 3000xG. After multiple washes to remove excess protein and a final resuspension with 100µl of sterile PBS plus 10mM Mg^{2+}, 25µl of either PBS plus 10mM Mg^{2+} (control) or 25µl of Proteinase K (Invitrogen) was added to a final concentration 200µg/ml. A final concentration of 5mM PMSF was added to stop the reaction and inhibit any other protease activity. After multiple washes in PBS plus 10mM Mg^2, the sample is boiled in 1x SDS loading buffer for 5 minutes. Samples were first normalized using Coomassie stained SDS PAGE gels and then normalized with a western blot to FlaB.

Buffer list

BSK2 2x Incomplete

BSK2 1x Complete

BSK2 2x Complete

50x TAE buffer- 242g/L Tris base, 57.1mL/L Glacial acetic acid, 100ml/L 0.5M EDTA (pH 8.0)

Polyacrylamide gel running buffer- 100mL/L 10x TG Buffer, 5ml/L 20% SDS

Nitrocellulose transfer buffer- 100mL/L 10x TG Buffer,

10x PBS- 80g/L NaCl, 2g/L KCl, 14.4g/L Na_2HPO_4, 2.4g/L KH_2PO_4

10x dPBS- 80g/L NaCl, 2g/L KCl, 11.5g/L Na2HPO4, 2.0g/L KH2PO4

EPS- 93g/L sucrose, 15% (v/v) glycerol

10x TG Buffer- 188g/L glycine, 30.2g/L Tris base

2x SDS Loading Buffer- 100mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromphenol blue, 20% (v/v) glycerol

6x DNA Loading Buffer- .25% (w/v) bromphenol blue, .25% (w/v) xylene cyanol FF, 30% (v/v) glycerol

10x TBS- 80g/L NaCl, 2g/L KCl, 30g/L Tris Base pH 7.4

10x TE- 100mM Tris-Cl, 10mM EDTA

Resolving Gel Buffer- 181.7g/L Tris base, 4g/L SDS pH 8.8

Stacking Gel Buffer- 60.6g/L Tris base, 4g/L SDS pH 6.8

Table 1. Plasmid List

Plasmid Name	Plasmid Origin	Antibiotic Marker(s)	Gene Description
pCspA	pKSSF1	SPC/STR	CspA
pOSK323	pCspA	SPC/STR	CspA tether missins amino acids 26-45
pOSK324	pCspA	SPC/STR	CspA tether missing amino acids 46-63
pJE1100	pOSK324	SPC/STR	CspA tether missing amino acids 42-63
pJE1101	pOSK324	SPC/STR	CspA tether missing amino acids 38-63
pJE1102	pOSK324	SPC/STR	CspA tether missing amino acids 34-63
pJE1103	pOSK324	SPC/STR	CspA tether missing amino acids 30-63
pJE1103h	pJE1103	SPC/STR	CspA tether missing amino acids 30-63 with a his tag
pJE1150	pRJS1009&pOSK324	SPC/STR	CspA w/OspA(wt) tether
pJE1150h	pRJS1009&pOSK324	SPC/STR	CspA w/OspA(wt) tether with a his tag
pJE1151	pRJS1089&pOSK324	SPC/STR	CspA w/OspA(dv21) tether
pJE1151h	pRJS1089&pOSK324	SPC/STR	CspA w/OspA(dv21) tether with a his tag
pRJS1098	pBSV2	KAN	OspA under constituative promoter
pJEctc	pRJS1098	KAN	OspA with C terminal cbp Tag
pJEctn	pRJS1098	KAN	OspA with N terminal cbp Tag
pJECCam	pJEctc & pSCv21x	KAN	OspA with C terminal cbp Tag and tet inducible CaM
pJENCam	pJEctn & pSCv21x	KAN	OspA with N terminal cbp Tag and tet inducible CaM
pJEWTCam	pRJS1098 & pSCv21x	KAN	WT OspA with tet inducible dv21 Cam
pJETPCam	pSCv21x & pXLF14301	KAN	homologous rec. with tet inducible dv21 Cam
pRJS1009	pBSV2	KAN	Surface localizing RFP
pRJS1089	pBSV2	KAN	Subsurface localizing RFP
pJElysm	pRJS1009&LysM&pBSV2.2	Gent	Surface localizing RFP with lysM domain from Borrelia

Table 2. Primer List

Primer Name	Primer Sequence
GL _tetR-rev	5’- GTGAAAGTGGGTCTTAATGAGGTACCGTAATCATGGTCATAGCTG -3’
GL _tetR-fwd	5’- TCATTAAGACCCACTTTCACATTTAAGTTG -3’
GR_Post-rev	5’- AAGTCCCAAAACTGGGACTTTTTTTAAATAAAAAAT -3’
GR_PflaB-fwd	5’- TCCCAGTTTTGGGACTGGGATCCTGTCTGTCGCCTCTTGTGGC -3’
G_OspA_CBPtag_C-fwd	5’-GTTTCTGCAGCTAATAGATTTAAGAAAATTTCTTCAAGTGGAGCTTTAT AATCTAGAGTCGACCTGCAGGCATG CAAGCTTGGCACTGGC -3’
G_OspA_CBPtag_C-rev	5’- TCTATTAGCTGCAGAAACAGCAATAAAATTCTTTTTCCACCTTCTTTTA GC ACCTGATCCACCAGGTTTTAAAGC GTTTTTAATTTCATC -3’
G_OspA_CBPtag_N-fwd	5’- GCAGCTAATAGATTTAAGAAAATTTCTTCAAGTGGAGCTTTACCTG GTG GATCAGGTGCTAGCGTTTCAGTAGA TTTGCCTGGTGAAATG -3’
G_OspA_CBPtag_N-rev	5’- TGAAGAAATTTTCTTAAATCTATTAGCTGCAGAAACAGCAATAAAATTC TTTTTCCACCTTCTTTTGTTTTTCTCG TCAAGGCTGCTAAC -3’
GL_OspA_CBPtagII-rev	5’- ACACTGATCCACCAGGGCTTTTAATTCCTGTGTATTCAAGTC -3’
GL_linkerCBPtag-fwd	5’- CCTGGTGGATCAGGTGCTAAAAGAAG -3’
GR_CBPtagII_OspA-fwd	5’- CCTGGTGGATCAGGTGCTGATGGATCTGGAAAAGCTAAAGAG -3’
GL_OspA_CBPtagI-rev	5’- CACCTGATCCACCAGGTTTGGAAGTTACTTTTTTTGATAC -3’
GR_CBPtagI_OspA-rev	5’- AGCACCTGATCCACCAGGTAAAGCTCCACTTGAAGAAATTTTC -3’
GR_CBPtagI_OspA-fwd	5’- TGGTGGATCAGGTGCTGACAAGTCATCAACAGAAGAAAAATT -3’
SOE OSPAwtTethCspa-fwd	5’- GTTAGCAGCCTTGACGAGAAAAACAGCTGTGATGAAAAAATTATGG AAAC -3’
SOE OspA21xTethCspa-fwd	5’- GCAGCCTTGACGAGAAAAACAGCTCTGATGAAAAAATTATGGAAACT ATCG -3’
SOE CspA_OspAwtteth-rev	5’- CTCGTCAAGGCTGCTAACATTTTGCTTGCATGAGGTGCAAATTAAAGT TAAAGTTAATATC -3’
SOE CspA_OspA21xteth-rev	5’- CTCGTCAAGGCTGCTATTTTGCTTGCATGAGGTGCAAATTAAAGTTAA TATC- 3’
pBSV2_5'Hind-fwd	5’- ACGTTCTAAAACGACGGCCAGTGC -3’
pBSV2_3'MCS-rev	5’- ACACTTTATGCTTCCGGCTC -3’
SOE-CspA_30-63_fwd	5’-CCTCATGCGCACCTTTTAGCTCTGATGAAAAAATTATGGAAAC-3’
SOE-CspA_34-63_fwd	5’-GCACCTTTTAGCAAAATCGATCCTTCTGATGAAAAAATTATGGAAAC-3’
SOE-CspA_38-63_fwd	5’- CGATCCTAAAGCAAATGCATCTGATGAAAAAATTATGGAAAC-3’
SOE-CspA_42-63_fwd	5’- GCAAATGCAAACACTAAGCCATCTGATGAAAAAATTATGGAA AC -3’
SOE-CspA_30-63_rev	5’- CCATAATTTTTTCATCAGAGCTAAAAGGTGCGCATGAGG -3’
SOE-CspA_34-63_rev	5’-CCATAATTTTTTCATCAGAAGGATCGATTTTGCTAAAAGGTGC-3’
SOE-CspA_38-63_rev	5’- CCATAATTTTTTCATCAGATGCATTTGCTTTAGGATGATCG -3’
SOE-CspA_42-63_rev	5’- CCATAATTTTTTCATCAGATGGCTTAGTGTTTGCATTTGC -3’
SOE-CspA_his_fwd	5’- CAGGTGCTCATCATCATCACCATCATTAATAGAAAGAAAAAAA ATAATATGTTG -3’
SOE-CspA_his_rev	5’- GATGATGATGAGCACCTGAGCCCCCGGGGTAAAAGGCAGGTT TTAAAGTATC -3’

BamCspAHis_rev	5’- GCGGATCCTTAATGATGGTGATGATGATGAGCACCTGAGCCCCCGGG GTAAAAGGCAGGTTTTAAAG -3’
BampostXLF14301_fwd	5’- GACGCGGATCCGCGTCGGGCAATTCCACCACACTGGACTAG -3’
Bampost_rev	5’- GACGCGGATCCGCGTCCTGGGACTTTTTTTAAATAAAAAATCTAC- 3’
Not1tetRXLF14301_rev	5’- GGGAACGTTGCGGCCGCAACGTTCCCTACCCGAGCTTCAAGGAA GATTTCC -3’
Not1tetR_fwd	5’- GGGAACGTTGCGGCCGCAACGTTCCCTCATTAAGACCCACTTTCACA TTTAAG -3’
mrfp1-lysM-rev	5’- AGCACCTGAGCCACCCGGGGCGCCGGTGGAG -3’
mrfp1-lysM-fwd	5’- CCGGGTGGCTCAGGTGCTGTAATAAAAATAGGCAATACCCTATG -3’
lysM-SphI-HindIII-rev	5’- TGCCAAGCTTGCATGCTCATTATTTGGCAGGAATTATTATCTTC -3’

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